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Insulin-Like Growth Factor II Promoter Expression in Cultured Rodent Osteoblasts and Adult Rat Bone*

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ABSTRACT

Insulin-like growth factor (IGF)-II stimulates bone formation by increasing the replication of cells of the osteoblastic lineage and by enhancing the differentiated function of the osteoblast. Although IGF-II is synthesized by skeletal cells, little is known about the mechanisms involved and its regulation by growth factors. IGF-II expression is tissue specific and is developmentally regulated. In the present study, we examined the expression of IGF-II in fetal rat, newborn mouse and MC3T3-E1 osteoblastic (Ob) cells, and in adult rat calvariae. We also determined mechanisms involved in the regulation of IGF-II by platelet-derived growth factor (PDGF) BB, fibroblast growth factor-2 (FGF-2), and transforming growth factor (TGF) β1. Northern analysis revealed IGF-II transcripts of 3.6 and 1.2 kb in osteoblastic cells and adult rat calvariae. Ribonuclease (RNase) protection assay using probes specific to the three known IGF-II promoters, P1, P2, and P3, demonstrated messenger RNA (mRNA) expression driven by P3 in osteoblasts and adult rat calvariae, but no expression of P1 or P2 transcripts. PDGF BB, FGF-2, and TGF β1 inhibited the expression of IGF-II P3 mRNA by 50%. PDGF BB, FGF-2, and TGF β1 also decreased the rates of IGF-II transcription in rat Ob cells as determined by nuclear run-on assays and did not modify the decay of IGF-II in transcriptionally arrested rat Ob cells. In conclusion, the synthesis of IGF-II in osteoblastic cells and in adult rat calvariae is driven by IGF-II P3 and is regulated by skeletal growth factors acting at the transcriptional level using the IGF-II P3. (*Endocrinology* **139:** 2287–2292, 1998)

INSULIN-LIKE growth factors (IGF)-I and -II are among the most prevalent growth factors synthesized by the osteoblast (1–5). IGF-I and IGF-II have modest mitogenic properties for skeletal cells, stimulate type I collagen synthesis, and decrease bone collagen degradation by inhibiting collagenase expression (6–8). The content of IGF-II in fetal rodent bone is 4 to 10 times higher than IGF-I (9). Although IGF-II is developmentally regulated and serum levels of IGF-II decline after birth, selected adult tissues, such as brain, heart, and bone continue to synthesize IGF-II (10–12).

Although the actions of IGF-II are similar to those of IGF-I, IGF-I is regulated by systemic hormones, whereas IGF-II is not (2, 13). PTH and GH stimulate IGF-I synthesis in osteoblasts, and cortisol is inhibitory (14, 15). The expression of both IGF-I and IGF-II is regulated by skeletal growth factors. Platelet-derived growth factor (PDGF) BB, fibroblast growth factor-2 (FGF-2), and transforming growth factor-β1 (TGFβ1) inhibit the expression of IGF-I and IGF-II, but the mechanisms responsible for the effect have not been defined (3, 16).

The rodent IGF-II gene is complex and consists of six exons. Exons 1, 2, and 3 are 5′-nontranslated leader exons, preceded by distinct promoters termed P1, P2, and P3 (17). Although the IGF-II gene structure is well conserved between human and rodents, the IGF-II human gene contains an adult, liver-specific promoter termed human P1. This promoter is responsible for IGF-II synthesis in adult human liver and is not expressed by rodent tissues (18). Since a homolog of the IGF-II human P1 is not present in rodents, the levels of circulating IGF-II in adult rodents are limited and derived from extra hepatic sources, possibly from skeletal, heart, and brain tissues. The rodent IGF-II gene is expressed as multiple messenger RNA (mRNA) transcripts ranging from 1.2–4.6 kb (17). This heterogeneity results from differential initiation of transcription of its promoters and the use of at least two sequential polyadenylation sites in the last exon. The IGF-II P3 initiates 3.6 and 1.2 kb mRNA, and it is the major promoter driving IGF-II expression in fetal tissues. Although the serum levels of IGF-II decline after birth, the concentrations of IGF-II in adult skeletal tissue remain significant. However, the promoter driving IGF-II expression and the mechanisms involved in the regulation of IGF-II in osteoblasts have not been established.

To characterize mechanisms involved in IGF-II gene regulation in rodent osteoblasts, we examined the expression of IGF-II and its promoters in adult rat bone, calvarial osteoblasts from newborn mice and 22-day fetal rats, and MC3T3-E1 cells, a mouse osteoblastic cell line. We also examined the mechanisms involved in the regulation of IGF-II by PDGF BB, FGF-2, and TGFβ1 in osteoblasts from 22-day fetal rat calvariae.

Materials and Methods

Culture technique

The culture method used was described in detail previously (19, 20). Parietal bones were obtained from 22-day-old fetal rats immediately after the mothers were killed by blunt trauma to the nuchal area, and from 3- to 5-day newborn mice killed by carbon monoxide (CO) asphyxiation. This project was approved by the Institutional Animal Care

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and Use Committee of Saint Francis Hospital and Medical Center. Cells were obtained by five sequential digestions of parietal bones from fetal rats and newborn mice, using bacterial collagenase (CLS II, Worthington Biochemical, Freehold, NJ). Cell populations harvested from the third to the fifth digestions were cultured as a pool and were shown to have osteoblastic characteristics (Ob cells) (19, 20). Cells were plated at a density of 5,000 to 10,000 cells/cm^2 and cultured in a humidified 5% CO_2 atmosphere at 37 C until reaching confluence (~50,000 cells/cm^2). For the nuclear run-on experiments, first passage of rat Ob cell cultures were used. For this purpose, cells were trypsinized, replated, and grown to confluence. Rat and murine Ob cells were cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Summit Biotechnologies, Fort Collins, CO). Passage 8 of immortalized osteoblastic MC3T3-E1 cells was plated at a density of 50,000 cell/cm^2 in αMEM (Life Technologies, Gaithersburg, MD) containing 20 mM HEPES and 10% FBS (21). Cells were grown to confluence at 37 C in a humidified 5% CO_2 atmosphere. Confluent Ob and MC3T3- E1 cells were exposed to serum-free medium for 20–24 h and then exposed to test or control medium in the absence of serum for 2–48 h. In 48-h treated cultures, the medium was replaced after 24 h with fresh control or test solutions. PDGF BB, FGF-2 (both from Austral, San Ramon, CA) and TGFβ1 (a kind gift of Genentech, South San Francisco, CA) were added directly to the medium. 5, 6-Dichlorobenzimidazole riboside (DRB) (Sigma Chemical Co., St. Louis, MO) was dissolved in absolute ethanol and diluted 1:200 in DMEM. For RNA analysis, the cell layer was extracted with guanidine thiocyanate at the end of the incubation and stored at −70 C. For the nuclear run-on assays, nuclei were isolated by Dounce homogenization.

Northern blot analysis

Total cellular RNA was isolated with guanidine thiocyanate, at acid pH, followed by phenol-chloroform extraction and ethanol precipitation or by RNeasy kit according to the manufacturer's instructions (Qiagen, Chatsworth, CA) (22). RNA from adult rat calvariae was obtained after pulverization of the bones and homogenization in a chilled guanidine thiocyanate solution. The RNA recovered was quantitated by spectrophotometry, and equal amounts of RNA from control or test samples were loaded on a formaldehyde agarose gel after denaturation. The gel was stained with ethidium bromide to visualize RNA standards and ribosomal RNA, demonstrating RNA loading of the various experimental samples. The RNA was then blotted onto Gene Screen Plus-charged nylon (DuPont, Wilmington, DE), and uniformity of the transfer was documented by revisualization of ribosomal RNA. A 1400-bp murine prepro-IGF-II complementary DNA (cDNA) (kindly provided by G. Bell, Chicago, IL) was purified by agarose gel electrophoresis (23). The IGF-II cDNA was labeled with [α-^{32}P]deoxycytidine triphosphate and [α-^{32}P]deoxyATP (50 μCi each at a specific activity of 3,000 Ci/mmol; DuPont) using the random hexanucleotide-primed second-strand synthesis method (24). Hybridizations were carried out at 42 C for 16–72 h, and posthybridization washes were performed at 65 C in 0.5 × saline-sodium citrate (SSC). The blots were stripped, rehybridized with a 752-bp *Bam*HI-*Sph*I restriction fragment of a murine 18S ribosomal RNA cDNA (American Type Culture Collection, Rockville, MD) and washed at 65 C with 0.1 × SSC. The bound radioactive material was visualized by autoradiography on Kodak X-OMAT AR films employing Cronex Lightning Plus intensifying screens (DuPont). Relative hybridization levels were determined by densitometry and corrected for the intensity of the 18S signal. Northern analyses shown are representative of three or more cultures.

RNase protection assay

Fragments containing sequences for the rat IGF-II promoter P1 (nucleotides −499 to +213), P2 (nucleotides −1164 to +140) and P3 (nucleotides −809 to +75), a kind gift from Dr. P. Holthuizen (Utrecht, The Netherlands) were subcloned from pSLA3 into pGEM 3Z (Promega, Madison, WI) (Fig. 1). The pTRIPLEscript vectors containing the sequences for the rat glyceraldehyde-3-phosphate dehydrogenase (GAPD) and ribosomal RNA (rRNA) 28S were obtained commercially (Ambion, Austin, TX). RNA was isolated as described for Northern analysis. Antisense RNA probes were generated by cloning DNA fragments into PGEM-3Z vector carrying an SP6 promoter in the presence of [α-^{32}P]uridine triphosphate and bacteriophage RNA polymerase SP6 (DuPont) (25). Total RNA was hybridized with 10^4 to 5×10^4 cpm of α-^{32}P-labeled antisense RNA probes to the IGF-II promoters P1, P2, P3, 28S, or GAPD constructs in a buffer containing 80% formamide at 45 C for 16 h. After hybridization, RNA samples were digested with RNase A and RNase T1 (Ambion) for 30 min at 37 C. Protected hybrids were extracted and precipitated with RNase inactivation/precipitation mixture (Ambion) and electrophoresed on an 8% polyacrylamide denaturing gel (Gel-mix 8, Life Technologies). The RNA-RNA hybrid was detected by autoradiography, and the relative hybridization levels were determined by densitometry and corrected for the intensity of the 28S signal. The sizes of the protected fragments were 213 bp, 140 bp, 75 bp, 316 bp, and 115 bp for IGF-II P1, P2, P3 (Fig. 1), GAPD, and 28S constructs, respectively, and were determined using pGEM DNA markers (Promega, Madison, WI).

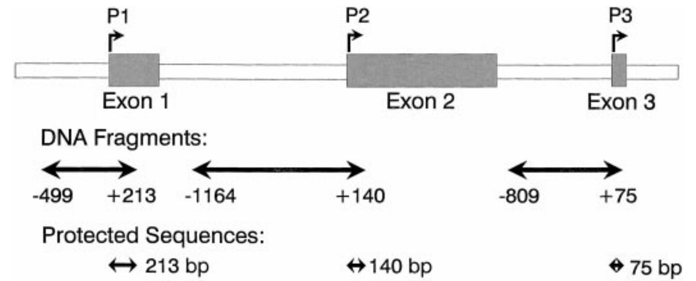

FIG. 1. Schematic representation of the rat IGF-II promoter region. *Arrows* indicate DNA sequences used to create riboprobes for the detection of IGF-II promoters (P) 1, 2, and 3. Protected fragments detected are indicated below.

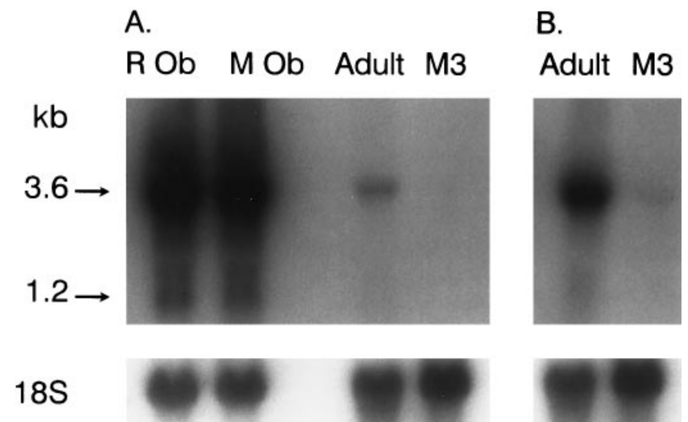

FIG. 2. A, Expression of IGF-II transcripts in primary rat (R) and mouse (M) Ob cells, adult rat calvariae (Adult), and MC3T3-E1 cells (M3). B, Expression of IGF-II mRNA in adult rat calvariae and MC3T3-E1 cells. Autoradiograph exposure was 10 times longer than in panel A. Eight micrograms of total RNA were extracted and subjected to Northern blot analysis and hybridized with α-^{32}P-labeled mouse IGF-II cDNA. The blot was stripped and rehybridized with a labeled mouse 18S cDNA. IGF-II mRNA was visualized by autoradiography and is shown in the *upper panel* while 18S ribosomal RNA is shown *below*.

Nuclear run-on assay

To examine changes in the rate of transcription, nuclei were isolated by Dounce homogenization in a Tris buffer containing 0.5% Nonidet P-40. Nascent transcripts were labeled by incubation of nuclei in a reaction buffer containing 500 μM each adenosine, cytidine, and guanosine triphosphates, 150 U RNasin (Promega), and 250 μCi [α-^{32}P]uridine triphosphate (3000 Ci/mM, DuPont) (26). RNA was isolated by treatment with DNase I and proteinase K, followed by phenol-chloroform extraction and ethanol precipitation. Linearized plasmid pBluescript SK^+ DNA containing about 1 μg of the IGF-II cDNA was immobilized onto Gene Screen Plus by slot blotting according to man-

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ufacturer's directions (DuPont). The plasmid vector pGL2-Basic (Promega) was used as a control for nonspecific hybridization, and murine 18S cDNA was used to confirm uniformity of the radioactive counts applied to each membrane. Equal counts per min of [α-^{32}P]-RNA from each sample were hybridized to cDNAs at 42 C for 72 h and washed in 1×SSC at 62 C for 30 min. Hybridized cDNAs were visualized by autoradiography, measured by densitometry, and corrected for the intensity of the 18S signal.

Statistical methods

Values are expressed as means ± SEM. Data on mRNA decay were plotted by linear regression, and the slopes obtained for control and treated cells were analyzed for significant differences using the method of Sokal and Rohlf (27).

Results

Northern analysis revealed IGF-II transcripts of 3.6 and 1.2 kb in rat and mouse Ob cells, adult rat calvariae, and an IGF-II transcript of 3.6 kb in MC3T3-E1 cells (Fig. 2). A 1.8-kb transcript was not detected by Northern analysis in Ob cells or adult rat bone, even after prolonged exposure of autoradiographs. IGF-II mRNA expression was of similar magnitude in rat and mouse Ob cells, whereas a lower level of expression was detected in adult rat calvariae, and minimal expression was detected in MC3T3-E1 cells. RNase protection assay revealed the presence of transcripts driven by the IGF-II P3 promoter in rat and murine Ob cells and adult rat calvariae (Fig. 3). Confirming the results obtained by Northern analysis, IGF-II P3 transcript expression was virtually undetectable in MC3T3-E1 cells (data not shown). IGF-II P1 and IGF-II P2 transcripts were not detected in either osteoblastic cells or fronto-parietal bone extracts.

Confirming prior observations, PDGF BB at 100 ng/ml, FGF-2 at 30 ng/ml, and TGFβ1 at 30 ng/ml inhibited IGF-II mRNA expression in mouse (not shown) and rat Ob cells (Fig. 4) (3). The effect was time dependent and after 48 h PDGF BB, FGF-2, and TGFβ1 decreased IGF-II mRNA levels by 70%. RNase protection assay demonstrated a time-dependent inhibition of IGF-II P3 mRNA expression in rat Ob cells after exposure to PDGF BB, FGF-2, and TGFβ1 (Fig. 5). The decrease was observed after 24 h and was sustained for 48 h. Densitometric analysis revealed that PDGF BB at 100 ng/ml, FGF-2 at 30 ng/ml, and TGFβ1 at 30 ng/ml for 48 h inhibited IGF-II P3 transcript expression by 79 ± 8%, 60 ± 16%, and 64 ± 5% (mean ± SEM; n = 4), respectively.

To determine whether PDGF BB, FGF-2, and TGFβ1 modified the stability of IGF-II mRNA, rat Ob cells were exposed to DMEM, PDGF BB at 100 ng/ml, FGF-2 at 30 ng/ml, or

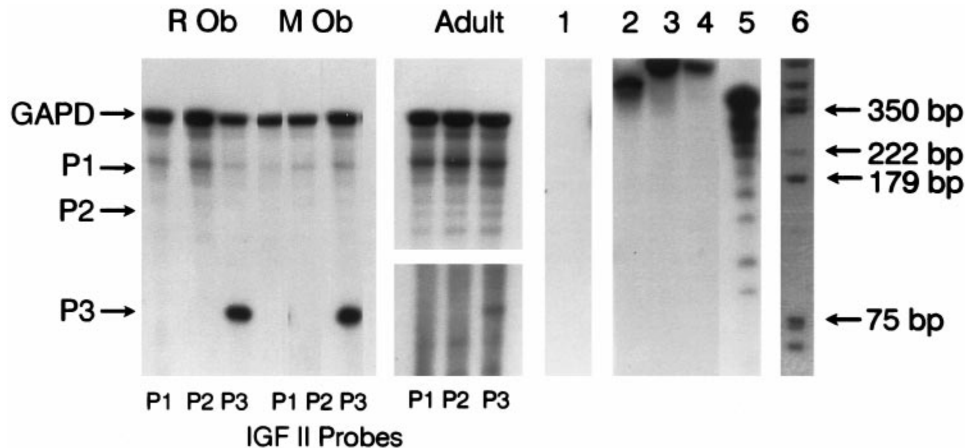

FIG. 3. Expression of rodent IGF-II promoter 1, 2, and 3 (P1, P2, and P3) mRNA in rat (R) and mouse (M) Ob cells, and adult rat calvariae (Adult). Ten micrograms of total RNA were extracted and subjected to RNase protection using α^{-32}P-labeled IGF-II P1 (P1) or IGF-II P2 (P2) or IGF-II P3 (P3) and GAPD probes. RNase-protected fragments were separated by PAGE, and the sizes of IGF-II P1 (213 bp), IGF-II P2 (140 bp), IGF-II P3 (75 bp), and GAPD (316 bp) protected fragments are indicated by *arrows*. Lane 1 shows a negative control (yeast transfer RNA); lanes 2, 3, 4, and 5 show IGF-II P1, P2, P3, and GAPD probes, respectively, and lane 6 shows pGEM standards. The *upper half* of the autoradiograph showing results of adult rat bone was underexposed in relationship to the *lower half* due to the intensity of the signal for GAPD.

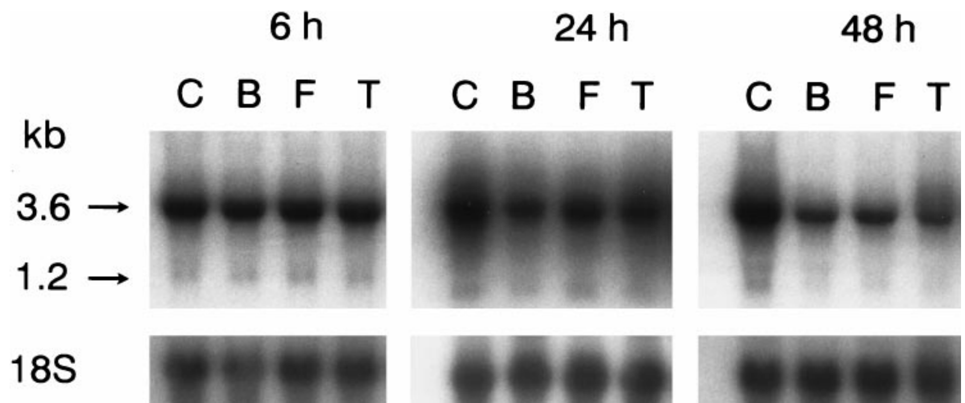

FIG. 4. Effect of PDGF BB (B) at 100 ng/ml, FGF-2 (F) at 30 ng/ml, and TGFβ1 (T) at 30 ng/ml on IGF-II mRNA expression in rat Ob cells treated for 6, 24, and 48 h. Total RNA from cultures exposed to DMEM (C), PDGF BB, FGF-2, or TGFβ1 was subjected to Northern blot analysis and hybridized with α-^{32}P-labeled mouse IGF-II cDNA. The blot was stripped and rehybridized with a labeled mouse 18S cDNA. IGF-II mRNA was visualized by autoradiography and is shown in the *upper panel* while 18S ribosomal RNA is shown *below*.

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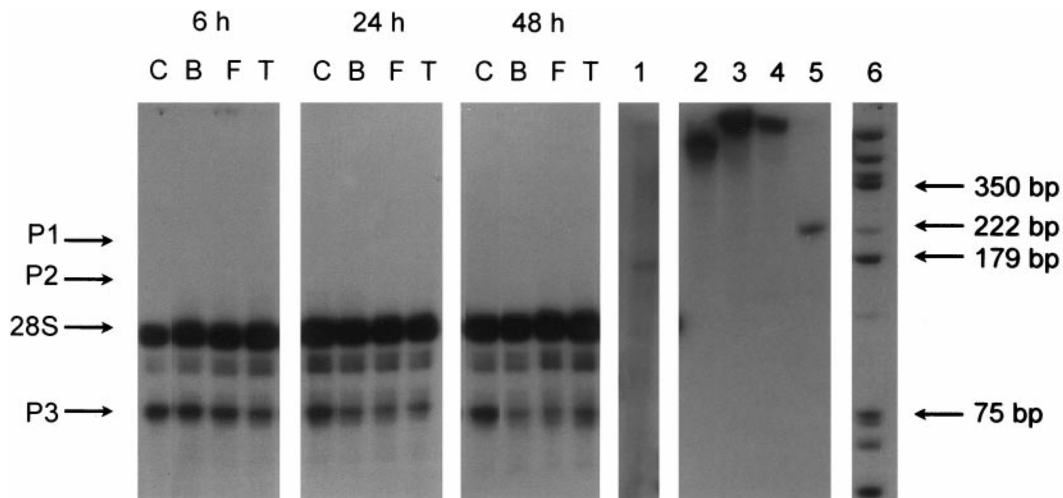

FIG. 5. Effect of PDGF BB (B) at 100 ng/ml, FGF-2 (F) at 30 ng/ml, and TGFβ1 (T) at 30 ng/ml on IGF-II promoter 1, 2, and 3 (P1, P2, and P3) mRNA expression in rat Ob cells treated for 6, 24, and 48 h. Confluent cultures of rat Ob cells were grown in serum-free medium for 24 h and exposed to DMEM (C), PDGF BB, FGF-2, or TGFβ1 for the indicated periods of time. Cells were harvested and 8 μg of total RNA were extracted and subjected to RNase protection using α-^{32}P-labeled IGF-II P1, IGF-II P2, IGF-II P3, and 28S probes. RNase-protected fragments were separated by PAGE, and the sizes of IGF-II P1 (213 bp), IGF-II P2 (140 bp), IGF-II P3 (75 bp), and 28S (115 bp) protected fragments are indicated by *arrows*. Lane 1 shows a negative control (yeast transfer RNA), lanes 2, 3, 4, and 5 show IGF-II P1, P2, P3, and 28S probes, respectively, and lane 6 shows pGEM standards.

TGFβ1 at 30 ng/ml for 1 h and then treated with 75 μM DRB, an RNA polymerase II inhibitor, in the absence or presence of growth factors for 6, 16, and 24 h (28). Longer exposure to DRB is not feasible in this culture model because of decreased cell viability beyond 24 h, as determined by trypan blue exclusion (E. Canalis, unpublished observations). In transcriptionally arrested Ob cells, the half-life of the predominant 3.6-kb IGF-II mRNA was estimated to be longer than 36 h by extrapolation of the values obtained at 24 h, and it was not altered by treatment with PDGF BB, FGF-2, or TGF β1 (Fig. 6). To confirm that PDGF BB, FGF-2, and TGFβ1 decrease IGF-II gene transcription, nuclear run-on assays were performed. For this purpose, nuclei were harvested from rat Ob cells cultured in the presence or absence of growth factors for 14, 24, and 48 h. When corrected for the 18S signal, PDGF BB at 100 ng/ml, FGF-2 at 30 ng/ml, and TGFβ1 at 30 ng/ml decreased the rate of transcription by 50 to 100% (Fig. 7).

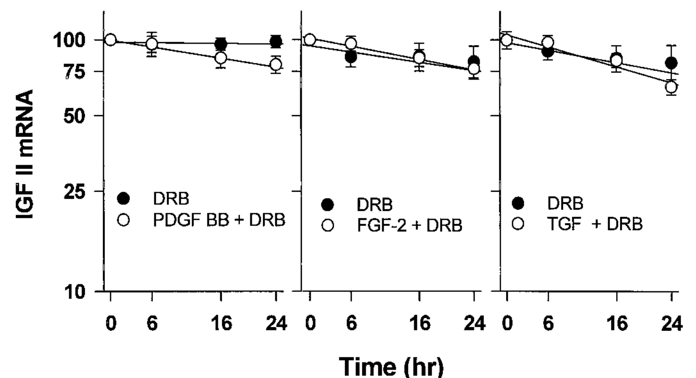

FIG. 6. Effect of PDGF BB at 100 ng/ml, FGF-2 at 30 ng/ml, and TGFβ1 (TGF) at 30 ng/ml on IGF-II mRNA decay in transcriptionally arrested rat Ob cells. Confluent cultures of rat Ob cells were grown in serum-free medium for 24 h and treated with PDGF BB, FGF-2, and TGFβ1 for 60 min before and 24 h after the addition of 5,6-dichlorobenzimidazole riboside (DRB). RNA was subjected to Northern blot analysis and hybridized with α-^{32}P-labeled mouse IGF-II cDNA, visualized by autoradiography, and quantitated by densitometry. Ethidium bromide staining of ribosomal RNA was used to check uniform loading of the gels and transfer. Values are means ± SEM for three to six cultures. Values were obtained by densitometric scanning and are presented as percentage of IGF-II mRNA levels relative to the time of DRB addition. Slopes were analyzed by the method of Sokal and Rohlf and were not found to be statistically different.

Discussion

This study was undertaken to compare the expression of IGF-II in fetal and adult rodent skeletal cells and to explore mechanisms underlying the regulation of IGF-II in osteoblasts. We demonstrated that rat and mouse Ob cells, MC3T3-E1 cells, and adult rat calvariae predominantly express an IGF-II transcript of 3.6 kb and that IGF-II expression in rodent osteoblasts and adult rat calvariae is driven by the IGF-II P3 promoter. Experiments in transcriptionally blocked cells using the RNA polymerase inhibitor DRB revealed that PDGF BB, FGF-2, and TGFβ1 did not modify IGF-II mRNA decay during a 24-h period. This, in conjunction with a change in the rate of transcription, indicates that PDGF BB, FGF-2, and TGFβ1 inhibit IGF-II at a transcriptional level. The results presented were generated in calvarial cells, and it is possible that the expression and regulation of the IGF-II gene is different in cells from long bones. The IGF-II gene has three different promoters of which P3 is the most active in extrahepatic tissues and cell lines in culture (17). IGF-II expression is tissue specific and developmentally regulated, so that most rodent adult tissues do not express this gene. However, bone, as well as heart and brain, express IGF-II (10, 17). The expression of IGF-II mRNA in MC3T3-E1 cells, a mouse osteoblastic cell line, was limited compared with primary cultures of mouse and rat osteoblasts. MC3T3-E1 cells

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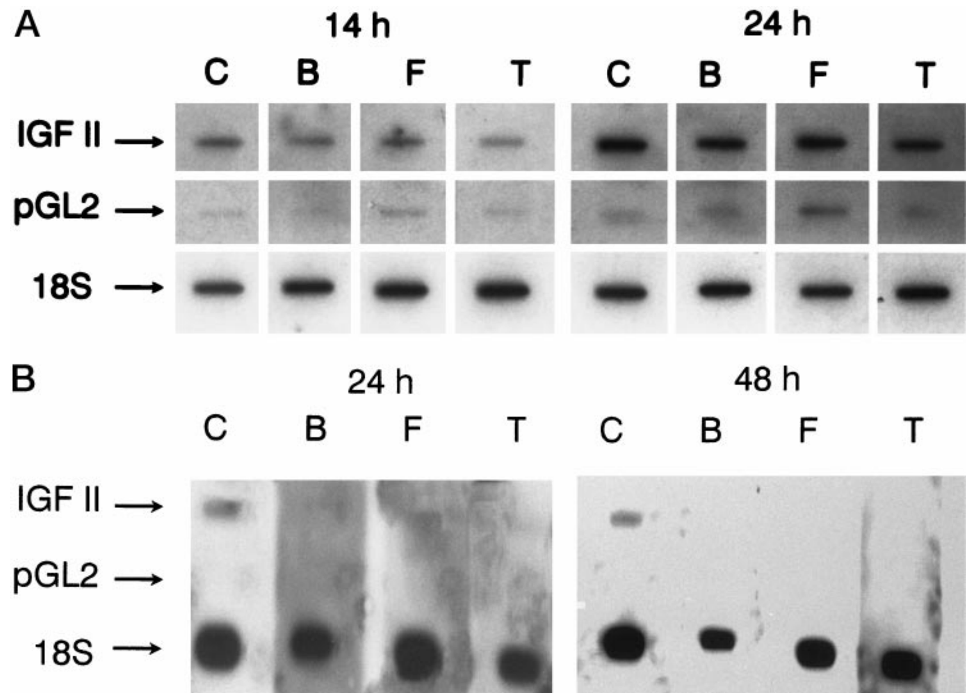

FIG. 7. Effect of PDGF BB (B) at 100 ng/ml, FGF-2 (F) at 30 ng/ml, and TGFβ1 (T) at 30 ng/ml on IGF-II transcription rates in cultures of Ob cells treated for 14 and 24 h (panel A) or for 24 and 48 h (panel B). Nascent transcripts from control (C) and treated cultures were labeled *in vitro* with [α-^{32}P]uridine triphosphate, and the labeled RNA was hybridized to immobilized linearized IGF-II cDNA. Murine 18S plasmid DNA was used to demonstrate loading, and pGL2-Basic vector DNA was used as a control for nonspecific hybridization.

unlike mouse Ob cells express more IGF-I than IGF-II (29). This could be explained by modifications of the cellular genotype after multiple cell passages resulting in changes in the expression of transcription factors necessary for the activation of the IGF-II gene. IGF-II regulation by PDGF BB, FGF-2, and TGFβ1 occurs through P3, the IGF-II promoter that is known to initiate transcription of IGF-II transcripts of 3.6 and 1.2 kb in size (17). Another study suggested that the 3.6-kb IGF-II transcript is likely to provide the major template for IGF-II synthesis and that the 1.2-kb transcript is generated by the use of alternative polyadenylation signals in the primary 3.6-kb transcript (30). Endonucleolytic cleavage of the abundant 3.6-kb mRNA may also occur and give rise to two 1.8-kb transcripts and provide an additional mode of regulating IGF-II production posttranscriptionally (31). However, 1.8-kb transcripts could not be detected in Ob cells. This confirms that expression of IGF-II in Ob cells and in adult rat calvariae and its regulation by growth factors occurs primarily at the transcriptional level and involves the IGF-II P3 promoter.

Although there are uncertainties about the physiological concentrations of PDGF BB, FGF-2, and TGFβ1, their effects were observed at doses that modify other parameters of metabolic function in Ob cells (32–34). This suggests that the inhibition of IGF-II is physiologically relevant. The inhibitory effects of growth factors on IGF-II synthesis may result in decreased collagen synthesis and increased collagenase expression. This may result in a decrease in bone matrix. In addition to decreasing IGF-II, skeletal growth factors modify the production of IGFBPs by the osteoblast. The inhibition of IGFBP-6 by TGFβ1 seems to be particularly relevant since IGFBP-6 binds IGF-II with higher affinity than IGF-I, and IGFBP-6 selectively blocks the effect of IGF-II on osteoblasts. The inhibition of IGFBP-6 may be a feedback mechanism to maintain adequate levels of IGF-II in bone (35). The IGF-II receptor may also act as a IGFBP and is not regulated by growth factors (E. Canalis, unpublished observations) (36, 37).

IGF-II is mostly abundant in fetal skeletal tissue. Studies on its direct actions in bone cells and studies in mice, either with targeted IGF-II gene disruption or overexpression of IGF-II, have documented its relevance to skeletal growth (38, 39). However, it also appears to play a role in the normal function of the adult skeleton. Bone is among the few adult tissues expressing IGF-II, and IGF-II levels in extracts of cortical human bone decline with aging, suggesting a possible role of IGF-II in the maintenance of bone mass and in the pathogenesis of osteoporosis (40).

In conclusion, our studies reveal that IGF-II is expressed by adult rat bone as well as by osteoblasts in culture, and its expression is driven by the IGF-II P3 promoter. Skeletal growth factors regulate IGF-II expression in osteoblasts acting at the transcriptional level.

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